DRAFT: August 31, 1994

DECISION DOCUMENT
TSCA SECTION 5(H)(4) EXEMPTION FOR PENICILLIUM ROQUEFORTI

I. SUMMARY

Penicillium roqueforti is a common saprophytic fungus that is widespread in nature and can be isolated from soil, decaying organic substances and plant parts. The major industrial uses of this fungus are for the production of blue cheeses, flavoring agents, antibacterials, polysaccharides, proteases and other enzymes. Most strains of P. roqueforti, including those used in cheese production, have been shown to be capable of producing a variety of mycotoxins. P. roqueforti's long history of use in the production of blue cheese has shown no adverse effects. Other industrial uses may, however, result in the production and release of certain mycotoxins. The potential risks from use of P. roqueforti in fermentation facilities are low.

II. BACKGROUND

A. Introduction

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA is proposing expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that would be exempt meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

B. Criteria for Minimizing Release from Manufacturing Facilities

The standards prescribed for the Tier I exemption require the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is proposing, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's proposed standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the bacteria used in fermentation processes are usually debilitated, either intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment.

Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is proposing that the number of microorganisms in fermentor gases be reduced by at least two logs prior to the gases being exhausted from the fermentor. EPA selected this number based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted in a gas from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also proposing that the requirements specify that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that entry be limited to essential personnel also addresses this

consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

C. Introduced Genetic Material Criteria

In order to qualify for either Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the

structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for metabolizing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform

the microorganisms proposed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should not prevent most microorganisms from meeting the exemption criteria, since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only if the introduced genetic material has been altered or selected to enhance frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the

introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with P. roqueforti, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of P. roqueforti will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of P. roqueforti, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of P. roqueforti, and EPA's review of the conditions selected.

D. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Penicillium roqueforti is discussed in the next unit.

III. EVALUATION OF PENICILLIUM ROQUEFORTI

A. History of Use

1. History of safe commercial use. The chief industrial use of P. roqueforti is in the production of Roquefort cheese. Strains of the microorganism are also used to produce compounds that can be employed in such uses as antibiotics, flavors and fragrances. While the fungus has been a constituent of Roquefort, Stilton and other blue cheeses and has been eaten by human since about 500 AD, there is evidence to indicate that most strains are capable of producing harmful secondary metabolites (alkaloids and other mycotoxins) under certain growth conditions. P. roqueforti is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules.

2. Products subject to TSCA jurisdiction. While EPA has not yet received a submission for a strain of P. roqueforti, some of the future uses of enzymes derived from P. roqueforti could be subject to TSCA. P. roqueforti can be used for the production of proteases and specialty chemicals, such as methyl ketones and 2-heptanone. Other strains of Penicillium species could be used in bioremediation. In these cases, the uses of the organism are likely to be subject to TSCA jurisdiction.

B. Identification of the Recipient Microorganism

1. Classification of the microorganism. Numerous studies have identified and classified Penicillium roqueforti at the genus, species and strain levels. The genus and species are considered to be well-defined on the basis of morphological features. Taxonomy for the genus Penicillium is governed mainly by morphological features, some of which are dependent on the medium used to culture the fungus. Therefore, strictly defined growth conditions are required for current taxonomy. Some taxonomists have suggested revising the series to which P. roqueforti belongs, to be based primarily on secondary metabolite production; however, this division has not yet been generally accepted. The taxonomy of some industrial strains may be unclear if they have undergone some mutagenesis and selection and do not conform to the taxonomy characteristics of the natural strains. However, given the considerable experience with these fungi, mycologists can now readily identify an isolate of Penicillium using standard media.

2. Related taxa of concern. Species closely related to P. roqueforti, on the basis of morphological characteristics, have been shown to produce antibiotics against certain strains of

bacteria. Principal among these is Penicillium notatum, which produces beta-lactams. However, the beta-lactams do not have widespread effects on microorganisms. There are also a few reported cases where closely related penicillia, such as P. chrysogenum have been found in association with infections.

C. Risk Summary

1. Studies regarding potential for adverse effects. The potential for pathogenicity of P. roqueforti even as an opportunistic pathogen is low. However, there is one documented case where P. roqueforti was found to cause hypersensitivity in a worker in a blue cheese manufacturing plant. Studies focusing on the potential adverse effects of P. roqueforti are based on toxicity of secondary metabolites, termed mycotoxins. Many of the strains of P. roqueforti isolated from commercial blue cheeses as well as from moldy grains and nuts have been shown in the laboratory to produce mycotoxins. Although there is a lack of documented cases of human toxicity, studies have shown that in the laboratory, industrial strains of P. roqueforti can produce mycotoxins. Some of the mycotoxins associated with P. roqueforti have been studied rather extensively but others are so newly described that they have received very little attention. PR toxin, the most potent of the P. roqueforti-associated mycotoxins, is unstable and deteriorates rapidly, so apparently under normal production conditions does not pose a health effects problem. Roquefortine, another of the more toxic mycotoxins, has been recovered from blue cheese at low levels; however there have been no reported adverse effects from consumption of the cheese.

P. roqueforti is not a known pathogen of plants or animals. The penicillia are responsible for the biodeterioration of stored grains and silage. Roquefortine and PR toxin produced in P. roqueforti have been implicated, but not documented, as the causal agent in instances of spontaneous bovine abortion and placental retention.

2. Studies regarding survival in the environment. P. roqueforti is saprophytic and is found normally in soil and decaying vegetation. Studies indicate that Penicillium species are able to utilize a number of carbohydrate and nitrogen sources and can grow over a broad pH (3-8) range.

IV. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. Penicillium roqueforti is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The Agency believes this exemption will result in

resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. In addition to assessing the risk of P. roqueforti, EPA has developed criteria limiting the potential for transfer of and expression of toxin sequences, and the conditions of use specified in the exemption are met (Tier I) or will be reviewed by EPA to ensure adequate risk reduction (Tier II). EPA requirements for minimizing numbers of viable microorganisms emitted are within standard operating procedures for the industry, and both the procedures and the structures specified in the exemption are the type industry uses to protect their products from contamination.

The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

V. RECOMMENDATION AND RATIONALE

A. RECOMMENDATION

Penicillium roqueforti is recommended for a TSCA section 5(h)(4) tiered exemption.

B. RATIONALE

1. Risks from use of the recipient microorganism P. roqueforti are low. P. roqueforti is generally considered to be a benign organism, but it does raise concerns because of its ability to produce mycotoxins under certain conditions. Despite these concerns, the organism has a history of use in the production of blue cheese without noted reports of adverse effects to workers or the environment. Most strains have not been documented to be serious pathogens of humans, animals, or plants. Cases involving mycotoxin production or allergic responses by workers exposed to P. roqueforti appear to be associated with a limited number of strains. Mycotoxin production is variable and depends on substrate composition and length of time and conditions of fermentation. Attention to these considerations contribute to controlling the amount and timing of exposure to mycotoxins in the industrial setting. Furthermore, setting the use of proper safety precautions, good laboratory practices, and proper protective clothing, allays concern for exposure of workers to mycotoxins produced by this

microorganism. Potential hazards to the public and the environment are mitigated by limitations to exposure brought about by the conditions of contained use which are designed to limit release of the microorganisms to the environment.

2. Risks from use of recombinant strains of P. roqueforti which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. Taxonomy of the Penicillium genus is complex and dependant on differences in morphological features. However, as part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using P. roqueforti. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

While production of certain mycotoxins has been associated with strains of P. roqueforti, companies have been using naturally occurring strains of P. roqueforti to produce blue cheese for many years without reports of toxic effects on workers. The limited in size constraints as well as the restriction on vertebrate toxins imposed on introduced DNA by the criteria for the section 5(h)(4) exemption should reduce the likelihood of increased production or exposure to mycotoxins potentially produced by P. roqueforti strains.

Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption. When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

Attachment 1:

INTEGRATED RISK ASSESSMENT FOR

PENICILLIUM ROQUEFORTI

I. INTRODUCTION

Penicillium roqueforti is a common saprophytic fungus, that is widespread in nature and can be isolated from soil, decaying organic substances and plant parts. The major industrial uses of this fungus are for the production of blue cheeses, flavoring agents, antibacterials, polysaccharides, proteases and other enzymes.

While the fungus has been a constituent of Roquefort, Stilton and other blue cheeses and eaten by humans since about 500 AD, there is considerable evidence to indicate that most strains are capable of producing harmful secondary metabolites (alkaloids and other mycotoxins) under certain growth conditions. (Peberdy, 1985; Sharpell, 1985).

History of Commercial Use and Products Subject to TSCA Jurisdiction

The chief industrial use of the fungus P. roqueforti, is in the production of Roquefort cheese. Strains of the microorganism are also used to produce compounds that can be employed as antibiotics, flavors and fragrances (Sharpell, 1985); uses not regulated under the Toxic Substance Control Act (TSCA).

The organism can also be used for the production of proteases and specialty chemicals, such as methyl ketones (Larroche et al, 1989) and 2-heptanone (Larroche and Gros, 1989; Jong and Gantt, 1987). Other strains of Penicillium species are also useful in biodeterioration (Peberdy, 1985). It is possible that because of its ability to survive in a variety of soil conditions, P. roqueforti could be used for bioremediation purposes. In these cases the uses of the organism are likely to be subject to TSCA.

II. IDENTIFICATION AND CLASSIFICATION OF THE MICROORGANISM

Fungi, in general, can be relatively difficult to identify or classify compared to other microbial groups such as the bacteria. Fungi are classified by morphological features that vary with cultural techniques and the experience of the taxonomist. Reliance on morphological characters may not serve as a dependable model for identification of closely related species. Molecular methods which are currently applied to bacteria have not advanced as rapidly with fungi. However, certain fungal genera, including Penicillium, can be classified with a fair degree of certainty by using standard media.

A. Definition of Penicillium roqueforti

Since the turn of the century, Thom (1910) and others have studied the genus Penicillium because of the importance of these fungi in the fermentation process of cheesemaking. Raper and Thom's "Manual of Penicillia" (1949) has been accepted for decades as the standard descriptive monograph. Raper and Thom (1949) placed the cheese-fermenting penicillia in two separate series, the P. roqueforti and the P. camemberti series. Given the considerable experience with these fungi, mycologists can now readily identify an isolate of Penicillium using standard media originally described by Raper and Thom (Alexopoulos and Mims, 1979). In practice, closely related strains with identical micromorphology were sometimes considered separate species (Samson and Gams, 1984). More recently, a species concept based primarily on morphological characters of conidiophores and conidia for P. roqueforti was adopted by Samson et al. (1977).

B. Taxonomic Characterization

P. roqueforti is traditionally identified by this organism's morphological characteristics and colony morphology when grown on specific growth media. Raper et al., (1968) describe colonies on Czapek's medium as broadly spreading, 5.0 to 6.0 cm in 10-12 days at room temperature, heavily sporing, velvety with surface fairly smooth or plane with broad, white, thin margin, cobwebby with hyphae radiating partly on the surface and partly just below the surface of the medium. Green conidial areas follow the hyphae in unevenly radiating lines. At margins, white shades into blue-green and various other shades of green. Reverse side is shades of green to bluish green, to almost black.

Morphology of the organism itself is based on features of the brush-shaped fruiting head; size, shape and number of conidia; size and number of sterigmata; whether there is branching; length and surface markings of the conidiophore;

overall dimensions; and like characters. Thus, examination of both colony morphology and microscopy wet mounts is necessary. While appearance may vary according to the medium on which cultures are grown, characteristics remain quite stable when subcultured on the same medium.

Numerous studies have identified and classified P. roqueforti at the genus, species and strain levels. The genus and species of P. roqueforti are considered to be well defined on the basis of morphological features. The predominant characteristics are the production of asexual spores in phialides with a distinctive brush-shaped configuration (Raper et al. 1944; Raper, 1957; Samson and Gams 1984).

Since Raper and Thom's work (1949), more than 70 additional species have been described for the genus Penicillium. Even today, the taxonomy is still governed mainly by morphological features. As these properties are relatively unstable under mutagenesis and selection, or long-term artificial culturing, the current taxonomy of some industrial strains may be difficult to ascertain.

Although the taxonomy of this group is related to a constancy of morphological features such as size and morphology of individual conidia, phialide shape and colony color (Raper and Thom, 1949; Pitt, 1979) some of these characteristics are dependent to an extent on the medium used to culture the fungus. Therefore, strictly defined growth conditions are required for the current taxonomy. Improvements in the taxonomy based on examining additional features such as physiological characters, DNA/DNA hybridization, ribosomal RNA sequences and the production of unique arrays of secondary metabolites are evolving, but not yet systematized (Samson and Gams, 1984).

Many of these Penicillium species either do not possess a sexual state (teleomorph) or it is rarely found and assumed to play a very minor part in their genetics in nature. According to Peterson (1990), no sexual state has ever been described for P. roqueforti. Fungi without sexual forms are placed in a taxonomic grouping called the fungi imperfecti (anamorph). At the present time P. roqueforti is in the fungi imperfecti grouping. Even though P. roqueforti has no reported sexual stage, it has been placed in the same taxonomic section with other imperfect penicillia that have been linked to the ascomycete teleomorph Eupenicillium (Peterson, 1990).

C. Related Species of Concern

For the reasons noted above with classification and identification of penicillia, it is frequently difficult to discriminate between closely related species. However, closely related species of this genus, based on morphological characteristics, produce antimicrobial chemicals (antibiotics) which can stop the growth of, or kill specific strains of bacteria. Principal among these is P. notatum which produces beta-lactams. However, these chemicals do not have wide spread effects on microorganisms. As discussed below, there are limited cases in which closely related penicillia are found in association with infections.

III. HAZARD ASSESSMENT

A. Human Health Hazards

Although the pathogenic potential of P. roqueforti is very low, even for an opportunistic pathogen, this fungus does on rare occasions cause hypersensitivity. There is only one documented report of infection in humans caused by P. roqueforti (Dynamac, 1991). Campbell et al. (1983) described a patient who worked in a plant where blue cheese was manufactured by use of P. roqueforti. This patient developed a cough, dyspnea, malaise, reduced lung volume and bibasalar crackles. A chest roentgenogram revealed bilateral infiltrates. Bronchoalveolar lavage fluid contained many lymphocytes and antibodies against P. roqueforti. Such antibodies were also present in the patient's serum.

There are limited cases in which closely related penicillia are found in association with infections. Peberdy (1985) in discussing the possibility of penicillia adopting the role of opportunistic pathogens in humans, mentions the report of Eschete et al. (1981) describing a case of P. chrysogenum as the cause of endopthalmis.

1. Toxins produced by Penicillium roqueforti and their toxicity

Many of the strains of P. roqueforti isolated from commercial blue cheeses as well as from moldy grains and nuts have been shown in the laboratory to produce mycotoxins (Jong and Gantt, 1987). These mycotoxins include isofumigaclavin C, penicillic acid, PR toxin, patulin, botryodiploidin and roquefortine. The effects noted with ingestion of these mycotoxins are mutagenesis and tumorigenesis as well as extensive

liver, kidney and nerve damage. Although there is a lack of documented cases of human toxicity, studies have shown that in the laboratory industrial strains of P. roqueforti can produce mycotoxins (Betina, 1989; Wei et al., 1985). However, the endpoints that are noted and the doses at which the effects are observed frequently are based on LD50 and omit references to No Observable Effect Level (NOEL) dosages. Finally, there is no assurance that the below noted data were derived from studies that employed Good Laboratory Principles.

Two of the toxins, roquefortine and PR toxin have vertebrate LD50 values of about 10 mg/kg intraperitoneal (CRC Handbook of Microbiology, 1987). This level of toxicity has routinely been considered "highly toxic" in EPA's evaluation of premanufacture notices (PMNs) on new chemicals. However, production of these toxins is related to the composition of the growth substrate and usually occurs in stationary phase cultures. While not universally true, mycotoxins are generally produced on high carbon/nitrogen solid substrates (Ciegler and Kurtzmann, 1970; Scott, 1984). The level of toxin production for specific cultures is variable but for research purposes can be induced to be as high as 1 mg/liter (Hohn, 1990).

Scott (1981) summarized these toxins and their synonyms, as well as their possible presence in blue cheese.

a. Roquefortine

Roquefortine is an indole mycotoxin. It is produced by P. roqueforti and some other Penicillium species, namely P. notatum, P. oxalicum, P. communi, P. corymbiferum, P. expansium and P. urticae (Scott, 1984). Roquefortine has been assigned the structure 10b-(1,1-dimethyl-2-propenyl)-3-imidazol-4-ylmethylene-5a,10b,11,11a-tetrahydro-2H-pyrazino-[1',2':1,5]pyrrol[2,3,b]indole-1,4-(3H,6H)-dione. (Scott and Kennedy, 1976). It is identical to roquefortine C.

Ueno and Ueno (1978) reported an intraperitoneal (IP) LD50 for roquefortine of 15-20 mg/kg in rats. Arnold et al. (1987) reported that roquefortine causes convulsive seizures when administered to mice IP in doses of 50-100 mg/kg (Scott et al., 1976). They reported LD50 of 169 mg/kg in male and 184 mg/kg in female CR57 mice and 189 mg/kg in male and 184 mg/kg in female Swiss-Webster mice. Neurologic properties reported by Scott et al., 1976, were not seen in the Arnold et al. (1987) study. However, Wagener et al. (1980) reported paralytic activity in day-old cockerels incubated with roquefortine.

Roquefortine was found to occur primarily in the mycelium of surface grown cultures of P. roqueforti. Independently, Scott et al. (1976) found roquefortine in yeast extract sucrose-grown mycelium of P. roqueforti. Low concentrations of roquefortine C were found in roquefort-type blue cheese by Ohmomo (1975), but exact concentrations were not reported. Scott and Kennedy (1976) found concentrations of roquefortine up to 6.8 mg/kg in samples of market blue cheese they examined. Ware et al. (1980) reported average levels of 0.42 µg/g of roquefortine in 12 samples of blue cheese and of 0.045 µg/g in two samples of blue cheese dressing. In fact, roquefortine seems to be produced by most strains of P. roqueforti isolated from blue cheese or used as cheese starters (Scott et al., 1977). A small percentage of strains recovered from meat also produce roquefortine (Leistner and Eckardt, 1979).

Schoch et al. (1984) conducted mutagenicity studies by the Ames test on six strains of P. roqueforti used commercially for the production of mold-ripened cheese. They also checked the six strains for roquefortine production and for mutagenic activity of the roquefortine. Neither the fungus or roquefortine showed any mutagenic activity by the Ames test (Schoch et al., 1983). Frank et al. (1977) fed 2.5 mL of a suspension of P. roqueforti and the cheese produced by the P. roqueforti once weekly to rats by gavage over their lifespan. They also gave subcutaneous injections of these suspensions once weekly subcutaneously for 52 weeks. There was no evidence of a possible carcinogenic effect.

Kough (1991) quotes the CRC Handbook of Microbiology, 1987, as showing roquefortine having an LD50 value of about 10 mg/kg which would place it among those substances considered "highly toxic" in the EPA's evaluation of chemicals under TSCA. An oral LD50 for roquefortine was not available. Frank et al. (1977) fed both a suspension of P. roqueforti and the cheese produced by the P. roqueforti to rats with no ill effect. They also gave these suspensions by subcutaneous injection without effect. However, the strains with which they worked had not been tested for toxin production. Scott (1981) believes "...no potential acute human health hazard can be extrapolated from the amounts of roquefortine present in blue cheese." However, until more is known about roquefortine, the amounts produced during commercial handling and its stability, it cannot be considered to be without some potential hazard to human and/or animal health.

b. PR Toxin and Eremofortines (and Derivatives)

PR toxin (7-acetoxy-5,6-epoxy-3,5,6,7,8,8a-hexahydro-3',8,8a-trimethyl-3-oxaspiro[naphthalene-2(1H,2'oxirane]-3'-carboxaldehyde) (Arnold et al., 1987) is one of the most acutely

toxic metabolites known to be formed by P. roqueforti (Scott, 1981). It is consistently detected, and frequently found in blue cheese (Leistner and Eckardt, 1979; Orth, 1976; Polonsky et al., 1980; Wei and Lui, 1985; Wei et al., 1976; Wei et al., 1973). Wei et al. (1973) isolated and partially characterized PR toxin from a strain of P. roqueforti recovered from toxic moldy feeds (later switching to an NRRL strain that proved to be a high producer). Following chromatography, the toxin could be detected by fluorescence under UV light. The median lethal dose of pure PR toxin IP in weanling rats was 11 mg/kg. The oral median lethal dose was 115 mg/kg. Within 10 minutes of an oral dose of about 10 mg (160 mg/kg) animals experienced breathing difficulties which persisted to death (Wei et al., 1973). Oral doses above about 130 to 160 mg/kg body weight were fatal to 60-g rats in 36 hours or less. Gross pathology consisted of swollen, gas-filled stomach and intestines, while histological changes included congestion and edema of lung, brains and kidney with degenerative changes in liver and kidney and hemorrhage in the kidney as well.

Chen et al. (1982) studied the toxic effects of PR toxin in mice, rats, anesthetized cats and preparations of isolated rat auricle. Toxic effects in mice and rats included abdominal writhing, decrease of motor activity and respiration rate, weakness of the hind leg and ataxia. Intraperitoneal LD50 in mice was 5.8 mg/kg. Mice, rats and cats injected IP developed ascites fluid and edema of the lungs and scrotum; IV injections caused edema of the lung and large volumes of pleural and pericardial fluids. LD50 in rats was 11.6 mg/kg IP and 8.2 mg/kg IV. Although arrhythmias occurred in the late shock stage, the contractile force of the isolated rat auricle was more affected than the heart rate. The investigators concluded that PR toxin produced acute toxic effects in animals via an increase of capillary permeability and direct damage to lungs, heart, liver, and kidneys.

Feeding maize silage infected with P. roqueforti to 112 dairy cows resulted in loss of appetite, cessation of rumen activity and gut inflammation (Vesely et al., 1981). First calves aborted in the 7th and 8th months. Sterile maize silage inoculated with P. roqueforti and incubated at 20EC produced up to 160 mg/kg PR toxin. Maximum production of 900 mg PR toxin/L occurred in liquid medium at 13EC after 50 days. A dose of 0.01 micrograms of PR toxin was extremely toxic to 40-h-old chicken embryos.

After 10-15 minutes, all weanling rats injected IP with 1.5 mg PR toxin developed breathing problems, motor incoordination and flaccid paralysis, particularly in the back legs (Polonelli

et al., 1978). Death ensued in 2-4 hours. Histological tests showed turbid swelling of hepatocyte cytoplasm. Intraperitoneal LD50 was 14.5 mg/kg body weight. Rats administered 0.5 mg PR toxin orally procapite/prodic for two months showed no visible effect. The intent to continue oral feedings was mentioned by Polonelli et al. (1978), but a review of the literature did not reveal published results. Mutagenicity of PR Toxin (1978) was demonstrated by Nagao et al. (1976) and Ueno et al. (1978) by the Salmonella typhimurium test and by Wei et al. (1979) by testing with Saccharomyces cerevisiae and Neurospora crassa.

Polonelli et al. (1982) carried out preliminary studies on possible carcinogenic effects of PR toxin in rats. They reported that 2 of 10 albino rats fed PR toxin developed tumors, i.e., one squamous cell epithelioma and one uterine sarcoma within 449 and 551 days, respectively. The control group developed one adenocarcinoma after a longer time span of 931 days.

Polonelli et al. (1978) also studied the conditions under which PR toxin is formed. They found PR toxin is produced only in stationary cultures, beginning on the 9th day of incubation, and increasing up to the 35th day, at which time it begins to decrease and disappears on approximately the 120th day. It is found only in the medium in which it is grown and within the pH range of 4.5-9.0. Toxigenesis occurred within the temperature range of 10E-30EC with the optimum temperature at 24EC. Toxin production was dependent upon the amount of sucrose in the medium, and began at 5% sucrose and reached a maximum at 15%. No PR toxin was formed under microaerophilic conditions. The authors speculate that microaerophilic conditions prevail in most cheeses, which could explain why PR toxin is not generally found in them. However, Arnold et al. (1987) pointed out that PR toxin reacts with ammonia and free amino acids present in high concentrations in blue cheese. PR imine and reaction products formed by mixing PR toxin with L-alpha-alanine or L-leucine were tested for toxicity. The acute toxicities of the PR derivatives were considerably lower than that of the parent compound. Scott and Kanhere (1979) noted similar phenomena. They conclude that both PR toxin and PR imine are unstable in blue cheese and believe that the agents responsible for destruction of PR toxin formed during ripening of the blue cheese are most likely amino compounds. However, they felt that more definitive experiments would be needed to assess any possible latent toxicological hazard from PR toxin, taking into account the cheese as a whole.

PR toxin enters into reactions involving its aldehyde function to form cross-links between DNA and protein (Moule et al., 1980). It also inhibits in vitro transcriptional capacity of nuclei isolated from the liver of male Wistar rats when the

compound is administered in vivo. The toxin inhibited both the RNA polymerase systems responsible for ribosomal RNA synthesis and heterogenous nuclear RNA synthesis (Moule et al., 1976). Lee et al. (1984) found that PR toxin inhibited the in vitro activities of rat liver DNA polymerases alpha, beta and gamma, as well. Hsieh et al. (1986) studied the effect of PR toxin in the mitochondrial HCO3-ATPase of the rat brain, heart and kidney. They concluded that of the three tissues tested, HCO3-ATPase of the heart mitochondria was most sensitive to PR toxin and that the HCO3-ATPase was inhibited in a noncompetitive, irreversible manner.

Dire et al. (1978) reported that P. roqueforti metabolites eremofortin A, eremofortin B, eremofortin C and eremofortin D, at 10 mg/mL had no effect on the ciliate protozoan C. campylum that they were using to detect toxicity. Moreau (1980) claimed that neither PR toxin or other derivatives of eremophilane, i.e., eremofortines, are found in cheese because of their instability. This was corroborated by Sieber (1978) who reported that PR toxin was isolated from P. roqueforti strains incubated on special media and also from P. roqueforti strains used for cheese manufacture. However, he found cheese ripening conditions did not favor production of the toxin.

c. Isofumigaclavine A and B

Isofumigaclavine A is another alkaloid produced by P. roqueforti. This toxin and the product of its hydrolysis, isofumigaclavine B, are identical with roquefortines A and B, respectively. These toxins were reported by Ohmomo and co-workers (1975, 1977) and by Kozlovskii (1979). Scott et al. (1977) reported yields of isofumigaclavine A determined over 7 to 35 days to be consistently low. These investigators tested P. roqueforti in 200 mL media for isofumigaclavine A production after 18 days incubation at 25EC. One strain of P. roqueforti did not produce detectable amounts of isofumigaclavine A in either the mycelia or the media. A second strain produced 0.5 and 0.1 mg in the mycelium and medium, respectively, in Medium I and 1.0 and 0.1 mg in mycelium and medium, respectively, in Medium II; a third medium did not produce detectable levels. However, when cultures were grown at 15EC instead of 25EC, 2 mg/mycelial mat of isofumigaclavine A formed, about three times that formed at 25EC. No isofumigaclavine A was detected in the medium; 0.06 mg is the limit of detection. In fact, isofumigaclavine A yields exceeded those of roquefortine in several commercial blue cheese samples (Scott and Kennedy, 1976).

It is of interest that blue cheese is generally ripened by storage at 9E-12EC for three months. Scott and Kennedy (1976)

found roquefortine in 16 of 16 samples of cheese from seven countries; isofumigaclavine A (mean 0.61 microgram/g) and traces of isofumigaclavine B were also usually present.

d. Dihydroroquefortine, Festuclavine and Marcfortine A (Alkaloids)

Some alkaloids produced by P. roqueforti are believed to serve as intermediates in the production of other alkaloids. Dihydroroquefortine, also known as roquefortine D, is described by Scott (1981) as "one of the two stereoisomeric 12,13-dihydroroquefortines." Roquefortine D is probably a precursor of roquefortine C (Ohmomo et al., 1975, 1977; Kozlovskii et al., 1979). Kozlovskii et al. (1979) reported isolating 3,12-dihydroroquefortine, a derivative of roquefortine. Festuclavine is a clavine alkaloid toxin produced by P. roqueforti. It was isolated and identified by Kozlovskii et al. (1979). Marcfortine A is a novel alkaloid, also obtained from P. roqueforti (Polonsky et al., 1980). Toxicological data on these chemicals is limited.

e. Mycophenolic Acid

Mycophenolic acid is a metabolite reported to be produced by all strains of P. roqueforti tested and by a few other species of penicillia (La Font et al., 1979). It has antibiotic activity against bacteria and dermatophytic fungi and also interferes with viral multiplication (Planterose, 1969). It has been used in the treatment of psoriasis (Marinari et al., 1977). The toxicity for mammals appears to be low: LD50 in rats is 2,500 mg/kg and 500 mg/kg IV; in mice the LD50 is 700 mg/kg and 450 mg/kg IV (Wilson, 1971). Chronicity tests of daily oral doses of 80 and 320 mg/kg for one year did not cause apparent signs of toxicity in rabbits (Adams et al., 1975). However, rats given daily oral doses of 30 mg/kg died within 9 weeks and rhesus monkeys receiving 150 mg/kg daily developed abdominal colic, bloody diarrhea, weight loss and anemia after two weeks (Carter et al., 1969). Thirty-five human patients who received high oral doses of mycophenolic acid (2.4 g to 7.2 g daily) for 52-104 weeks had some adverse reactions, including cramps, nausea and diarrhea (Marinari et al., 1977). Scott (1981) reported that Umeda et al. (1977) induced mutations and chromosome aberrations in a mouse mammary carcinoma cell line with mycophenolic acid, but the compound was not mutagenic in Salmonella systems (Nagao et al., 1976; Webner et al., 1978). La Font et al. (1979) checked 16 strains of P. roqueforti for mycophenolic acid using four media to test production, thin-layer chromatography for assays and chicken embryos for toxicity tests. All strains produced mycophenolic acid, some on the order of 0.8 to 4 mg/g of dry culture. Greatest yields were obtained after 10

days of incubation at 15EC. La Font et al. (1979) mention studies (unpublished) using fluorodensitometric assays for mycophenolic acid in marketed blue-mold cheeses; 38% of studied samples were positive with 3% of the cheeses having levels of mycophenolic acid higher than 10 mg/kg. Strain differences in the P. roqueforti as to the amount of mycophenolic acid produced were noted.

Engel et al. (1982) did not find that all strains of P. roqueforti produced mycophenolic acid. They found that out of 80 strains, 20 were able to produce up to 600 mg in 2% yeast extract-5% sucrose broth. Sixty-two of the strains had been recovered from starter cultures of blue-veined cheeses from western Europe. Only seven of these 62 produced mycophenolic acid. All of the producer strains came from an individual; and in the market, cheeses with mycophenolic acid as high as 5 mg/kg of mycophenolic acid were only found in samples from this same factory. Toxicity tests in this study were performed with Detroit 98 and Girardi Heart human cell lines and one established pig kidney cell line (Am II). Schoch et al. (1983) did not detect any mycophenolic acid in the six strains of P. roqueforti they cultivated on semi-synthetic medium.

The oral LD50 of 700 mg/kg in mice placed mycophenolic acid in EPA's moderately toxic category.

f. Patulin, Penicillic Acid and Citrinin

Although there have been surveys in cheeses for the toxic metabolites patulin, penicillic acid and citrinin; they have not been found. Nonetheless, they are known metabolites of P. roqueforti. Olivigni and Bullerman (1978) reported the production of patulin and penicillic acid by an atypical P. roqueforti isolated from cheddar cheese. The culture extracts were toxic to Bacillus megaterium and chicken embryos. Commercial strains of P. roqueforti used to produce blue cheeses were not shown to produce these metabolites. Moubasher et al. (1978) found penicillic acid in two of six strains of P. roqueforti recovered from blue cheese, and Leistner and Eckardt (1979) in one of 80 strains isolated from food and grains. Scott (1981) reviewed other recoveries of penicillic acid: Karow et al. (1944) obtained it from P. suavolens (synonym for P. roqueforti) and Samsen et al. (1977) from fermented cheese. Seven isolates of P. roqueforti isolated from moldy grapes all produced patulin after 9 days at 25EC in yeast extract-2% sucrose-15% medium. Amounts varied from 20-1267 micrograms/5 mL cultures. Six cheese isolates produced no patulin under these conditions. One isolate from fresh grapes produced patulin. One isolate from meat produced both patulin and the nephrotoxin

citrinin; the other two isolates produced patulin only (Scott, 1977).

The available toxicological data on these chemicals is limited. Scott (1977), from results of subcutaneous injections of rodents, reported that these two chemicals may have carcinogenic capabilities, but a long-term oral feeding of rats gave no such indication (Osswald et al., 1978).

It is apparent that patulin and penicillic acid are not frequently formed by P. roqueforti (though they may be more common in moldy cheese that perhaps has been stored too long). They are also unstable in cheese (Lieu and Bullerman, 1977). This all suggests that the health hazards posed by these two substances are slight.

Stability of citrinin is uncertain in moist grains (Mintzlaff and Machnik, 1972). Little work appears to have been done with this toxin, perhaps because it has not been among the metabolites that P. roqueforti produces in cheese.

g. Botryodiplodin

Botryodiplodin has been reported as a mycotoxin synthesized by P. roqueforti. Moulé et al. (1981) reported that this toxin inhibited cell multiplication in growing cell cultures at concentrations without effect on cultures nearing or at confluence. In the growing culture the toxin affected DNA, RNA and protein synthesis. Moulé et al. (1982) further showed that botryodiplodin induces DNA-protein cross-links in rat hepatoma cells and hamster lung fibroblasts. Botryodiplodin was not among the mycotoxins detected in the six P. roqueforti strains isolated from mold-ripened cheese (Schoch et al., 1983).

h. Siderophores, Betaines and "Other" Toxins

Scott (1981) summarizes the other possible toxic metabolites produced by P. roqueforti as: ferrichrome, which was found in cheese together with an unknown negatively charged siderophore, which had 5-10 microgram/g siderophore activity (it is speculated that siderophores in food may complex iron, making it unavailable for bodily use); coprogen which is not found in cheese, and about which little appears to be known; water-soluble betaines, ergothioneine and hercynine, also about which little is known; toxins "1, 2, and 3", the last two of which had weak acute toxicity for mice. Scott (1981) states that there are reports of toxigenic P. roqueforti strains recovered from chestnuts, pecans and meat products.

i. Combined Effects of Toxins

No reports were found that deal with possible combined toxin effects as they might occur in a product.

j. Summary

Health effect concerns for this organism lie with its production of a variety of mycotoxins, some of which have been studied rather extensively and some of which are so newly described that they have received very little attention. Some of these mycotoxins have been shown to be produced by P. roqueforti strains used for cheese production and some have been detected in small amounts in the cheese itself. PR toxin and roquefortine appear to be the most toxic of the mycotoxins produced by P. roqueforti. PR toxin, one of the most potent mycotoxins, is unstable and deteriorates rapidly, so apparently under normal production conditions does not pose a health effects problem. Roquefortine has been recovered from blue cheese at low levels and there have been no reported adverse effects from consumption of the cheese.

The composition of medium used to make cheese and the length of time and conditions of the fermentation lead to highly variable results with respect to the composition and amounts of mycotoxins produced. In general, mycotoxins are produced in media with a high carbon to nitrogen ratio. The production of mycotoxins in TSCA-related usage is less likely as the production of specialty chemicals is expected to occur over significantly shorter timeframes compared with the fermentation of cheese. Under these conditions the production of mycotoxins during fermentation for specialty chemicals is anticipated to occur at lower levels, if at all, compared with the production of cheese.

B. Environmental Hazards

1. Plant and Agriculture Hazards

P. roqueforti is not a known pathogen of plants. Penicillium species are known to cause the deterioration of stored agricultural products. The species P. expansum, P. digitatum and P. italicum are responsible for significant losses of stored citrus, apples and pears (Peberdy, 1985). All but 3 of the strains of P. roqueforti listed for distribution by the ATCC require a USDA permit due to their ability to degrade seeds and plant products (Singh, 1990). However, there are no known published reports which document P. roqueforti as infecting plants.

2. Animal Hazards

P. roqueforti is not a known pathogen of animals. Penicillia are saprophytes that play an important role in cycling organic substrates. The penicillia are also responsible for the biodeterioration of stored grains and silage. Many fungal species including P. roqueforti, have been shown to be capable of producing toxins in stored grain and silage. PR toxin and roquefortine produced in P. roqueforti molded feed grain have been implicated, but not documented as the causal agent in instances of spontaneous bovine abortion and placental retention (Wei et al., 1973; Moreau and Moss, 1979; Haggblom, 1990) as other toxin producing fungal strains were present. There are no known published reports which document P. roqueforti as infecting animals. Indeed there are few known reports of any Penicillium species causing infection in an animal.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

P. roqueforti is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986).

No data were available for assessing the release and survival specifically for fermentation facilities using P. roqueforti. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially

greatest, ie. near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

P. roqueforti is saprophytic and is found normally in soil and decaying vegetation. Reproduction is asexual and involves the production of conidia. The genus is aerobic, but the oxygen requirements needed for growth have not been determined. Penicillium species are able to utilize a number of carbohydrate and nitrogen sources and can grow over a broad pH (3-8) range (Peberdy, 1985). These properties make it likely that any released P. roqueforti strains would survive in the environment. It has been reported that Penicillium species degrading decaying vegetation release nutrients that favor growth of mycobacteria by selectively inhibiting microbial antagonists (Henis, 1987).

2. Releases

Estimates of the number of P. roqueforti organisms released per production batch are tabulated in Table 1. The minimally controlled scenario assumes no treatment of the fermentor off-gas and assumes 100-fold (2 log) reduction of the maximum cell density of the fermentation broth resulting from inactivation (Reilly, 1991). The containment criteria required for the full exemption scenario assume the use of in-line filters to treat vent gases and a 99% removal efficiency under normal operating conditions. They also assume an overall 6-log reduction relative

to the maximum cell density of the fermentation broth resulting from inactivation steps (Reilly, 1991).

TABLE 1. Estimated Number of Viable Penicillium roqueforti Organisms Per Production Batch

Release Media	Minimally Controlled (cfu/day)	Full Exemption (cfu/day)	Release (days/year)
Air Vents	$2x10^{8}$ - $1x10^{11}$	$2x10^{6}$ - $1x10^{9}$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{12}$	$7x10^{8}$	90
Soil/Landfill	$7x10^{14}$	$7x10^{10}$	90

Source: Reilly, 1991

3. Air

While there is no specific information on the survival of P. roqueforti in the atmosphere, the organism's saprophytic nature and ability to form spores suggests that survival rates would be very good. Environmental exposure would occur as the organisms drift to earth and take up residence in the soil. Human exposure is expected to be low, since the numbers of organisms released would be quickly diluted in the atmosphere (LaVeck, 1991).

4. Water

P. roqueforti released to water would be expected to survive publicly owned treatment works (POTW) treatment and discharge. Surface water concentrations of organisms were estimated using the 10% and 50% flow values for SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals) that release to surface water. The SIC code flow was estimated using 128 indirect (facilities that send their waste to a POTW) and direct (facilities that have an NPDES permit to discharge to surface water) dischargers. Discharger data were extracted from the IFD (Industrial Facilities Dischargers) database and surface water flow data were taken from the RXGAGE database, maintained by the EPA. These data, which were partitioned into percentile rankings and flows for the 10th percentile (small river) and 50th (average river), were extracted and used for the exposure calculations. Flow is expressed in Millions of Liters/Day (MLD). Mean Flow is the average flow value, and 7Q10 flow is the lowest flow observed over 7 consecutive days during a 10 year period. Concentrations of microorganisms in surface water are calculated for both the

minimally controlled and the full exemption scenarios (LaVeck, 1991).

TABLE 2. *Penicillium roqueforti* Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	Q710	Mean	Q710
Minimally Controlled				
10th Percentile	159	4.57	4.4×10^{4}	1.53×10^{6}
50th Percentile	768	68.13	9.11×10^{3}	1.03×10^{5}
Full Exemption				
10th Percentile	159	4.57	4.4×10^{0}	1.53×10^{2}
50th Percentile	768	68.13	9.11×10^{-1}	1.03×10^{1}

*MLD = million liters per day
Source: LaVeck, 1991

5. Soil

Since soil is a natural habitat for *P. roqueforti*, long term survival is expected. The discharge area should become established with the released organisms. These releases could result in human and environmental exposure. *P. roqueforti* that is landfilled would not survive as well, since the anaerobic conditions in landfills could result in cell death. If the organisms were spread out over the surface of the soil, then survival would be enhanced. If any *P. roqueforti* became established in decaying vegetation, growth of mycobacteria could be enhanced (LaVeck, 1991).

V. INTEGRATION OF RISK

A. Discussion

1. Characterization of the Organism

P. roqueforti is a ubiquitous, saprophytic fungus frequently found on decomposing organic material. As with all fungi the conventional means of identification is based on morphological characteristics. This is in contrast to bacterial systematics, which rely on biochemical tests that produce qualitative responses and standardize the identification of the organism.

Given the long history of use of P. roqueforti in microbial fermentations, the typical source of strains for industrial uses today would be standard culture collections.

P. roqueforti is principally used in the production of cheeses, a non-TSCA application. TSCA applications include the production of enzymes and specialty chemicals through fermentation processes. Also, there is the possible application of P. roqueforti for bioremediation processes. The utility of this organism for microbial fermentation uses is well established.

2. Risks to Humans

P. roqueforti is a benign, nonpathogenic organism. Among the literature reviewed for this assessment, there has been only one reported case of pathogenicity. There are anecdotal reports of abortion in cattle brought about by the consumption of feed contaminated with P. roqueforti, although the correlation with disease is not strong. Contaminated feed can be assumed to be colonized by a variety of microorganisms which may produce toxins. There is no report of associating P. roqueforti with abortion in cattle through Koch's Postulates. Moreover, the relevance of these reports to human health issues is questionable.

The primary potential human health effect of P. roqueforti is the production of mycotoxins. The most toxic of these are roquefortine and PR toxin. Other mycotoxins produced by this organisms appear to be less toxic and of low concern. Health effects data on PR toxin and roquefortine are based principally on animal data. An LD50 in rats has been reported as 10-20 mg/kg intraperitoneal. The available data on exposure to roquefortine and PR toxin appear to be limited to food consumption. Roquefortine has been recovered from blue cheese at low levels and there have been no reported adverse effects from consumption of the cheese.

PR toxin has been shown to cause decreased motor activity and respiration rates, and hind leg weakness in mice and rats. It has also been shown to be lethal in rats and mice at relatively high intraperitoneal doses. Similar to roquefortine, PR toxin has been recovered from cheese; however, data indicate that PR toxin is unstable in cheese presumably accounting for the absence of adverse effects in humans from consumption of this cheese.

Conditions conducive to the production of mycotoxins by P. roqueforti include a medium of high C/N ratios (usually with the

medium supplemented with sucrose), growth of the fungus on the surface of the medium presumably due to the high oxygen content, and growth of the fungus in stationary phase. The first two condition may most likely be encountered during a commercial fermentation process.

Under fermentation conditions, the C/N ratio of the medium will be tailored to the need of the fungus based on its nutritional requirements. In general, microorganisms are most productive during the early phases of the growth stage when conditions are conducive to vigorous growth (i.e., when metabolism is highest, nutrient level is greatest, and cellular waste is lowest). Fungal fermentations, in some cases, have extended periods of surface/air interface cultivation; a condition conducive to the production of mycotoxins. However, the uses of P. roqueforti under TSCA are primarily expected to include the production of specialty chemicals. Microbial fermentation for the production of specialty chemicals under TSCA have a significantly shorter fermentation period (days or weeks) when compared to typical periods for cheese production (months). Shorter fermentation periods are less likely to result in stationary phase growth of the fungus. Finally, the production of toxins vary between strains of P. roqueforti: under specified conditions some strains produce mycotoxins while others do not.

P. roqueforti is classified as a class containment 1 microorganism under the NIH Guidelines and is therefore Good Large Scale Practices containment criteria designed to limit potential exposure to either the microorganism or its products. This limited exposure allays concern for exposure of either workers or the public to mycotoxins produced by this organism. The unstable nature of the PR toxin further reduces concern for exposure of workers or the public to this mycotoxin. Overall, this organism has a history of safe use without noted reports of adverse human health effects.

3. Risks to the Environment

Effects to nonhuman targets remain low. The concern for effects on cattle from the consumption of stored silage is based on anecdotal evidence. Effects were noted to occur following consumption of moldy silage. The residue contained, among other organisms, P. roqueforti. However, a more definitive test such as Koch's Postulates, was not carried out to determine the causative agent. This organism is not known to be a pathogen of plants.

Potential environmental hazards are mitigated by limitations to exposure brought about by the conditions of contained use.

The containment conditions and practices employed in industrial microbial fermentations are designed to limit release of the organism to the environment.

B. RECOMMENDATION

P. roqueforti is recommended for the tiered 5(h)(4) exemption.

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